

THIRD ANNUAL REPORT

February 1, 1994 - January 31, 1995

"Evaluation of Dried Storage of Platelets for Transfusion:
Physiologic Integrity and Hemostatic Functionality"

Grant No. N00014-92-J-1244
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Marjorie S. Read, Ph.D., Robert L. Reddick, M.D., Co-Investigators

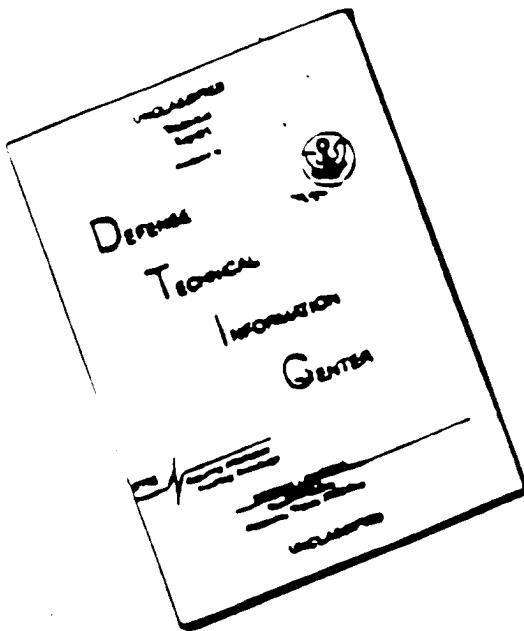
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Attachments:

1. Report from subcontract Principal Investigator, Marjorie S. Read, Ph.D., The University of North Carolina at Chapel Hill
2. Abstracts from ASH (3), AABB (1), and BBTS (1)

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ADMINISTRATIVE ACTIVITY:

Patents: The patent application protecting our process of stabilizing and freeze-drying human platelets for transfusion is still under review in the US Patent Office and has been submitted under the Patent Cooperation Treaty in all PCT member countries, also in Mexico. Armour Pharmaceutical Corp. has exercised its licensing option and has begun developing a large-scale manufacturing process to produce platelet preparations comparable to ours. Both ECU and UNC are assisting in the technology transfer.

Publications: Our first paper on the lyophilized platelets was published in the Proceedings of the National Academy of Sciences (vol. 92:397-401, 1995). This paper was of broad scope and included both in vitro and in vivo experimentation. A second paper was submitted in November to the journal Blood, but was not accepted for publication at this time. The PNAS paper and the submitted Blood manuscript will be made available upon request. Some elements of the data contained in these papers are also presented in this report. An abstract given by the PI in oral presentation at the Annual Meeting of the American Association of Blood Banks (Nov. 12-17, 1994; San Diego, CA) included data generated under N00014-92-J-1244 and N00014-93-1-1034. Three abstracts presented by poster at the annual meeting of the American Society of Hematology (Dec. 2-6, 1994; Nashville, TN) also contained data from both projects. An overview of the entire project with summation of in vitro and in vivo data to-date was given by the PI by invitation at the Annual Meeting of the British Blood Transfusion Society in Southampton, England, Sept. 13, 1994. A lay description of the project and its progress was published this year in the Durham Morning Herald and the North Carolina Beacon newspapers. Local Grand Rounds and departmental seminars also were given on project data at both ECU and UNC-Chapel Hill. Several other manuscripts and abstracts are in preparation relevant to the continuing activities of this grant.

Goal Completion: The majority of work is completed on each of the seven original specific aims. In Year One, Aim #4 (metabolic studies of rehydrated platelets) was modified due to departure of the relevant collaborating investigator from ECU; instead, Aim #1 was strengthened (tests of hemostatic efficacy) by adding Morris Blajchman's thrombocytopenic rabbit model as a test of hemostasis to apply to the lyophilized platelets. This year much emphasis was given to this model, and the data generated further proved the hemostatic potential of our human lyophilized preparations. See below for specific scientific progress summaries.

A no-cost extension of the project was approved with a new termination date of 06/30/95. This request was made to allow time to finish up the long term dried storage studies and to complete more testing in the in vivo hemostasis and circulation animal models. The new termination date also coincides with the University's fiscal year.

Armour Pharmaceutical Corporation has begun to produce platelets by our procedure in preparation for an IND application to the FDA slated for 1996. This collaboration signifies a great likelihood that freeze-dried platelets will become a reality

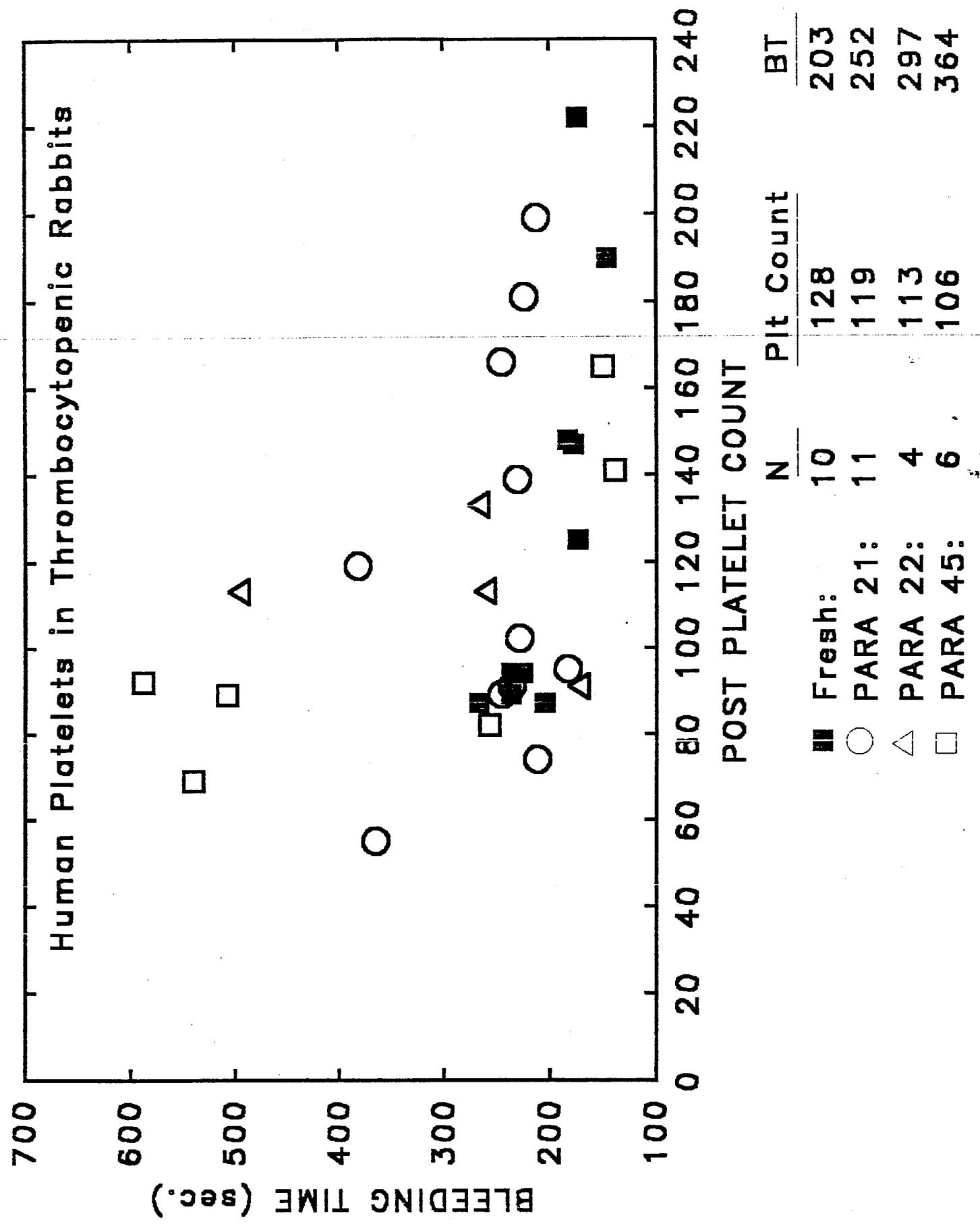
in the development of new medical resources for support of combat casualty care.

SCIENTIFIC PROGRESS:

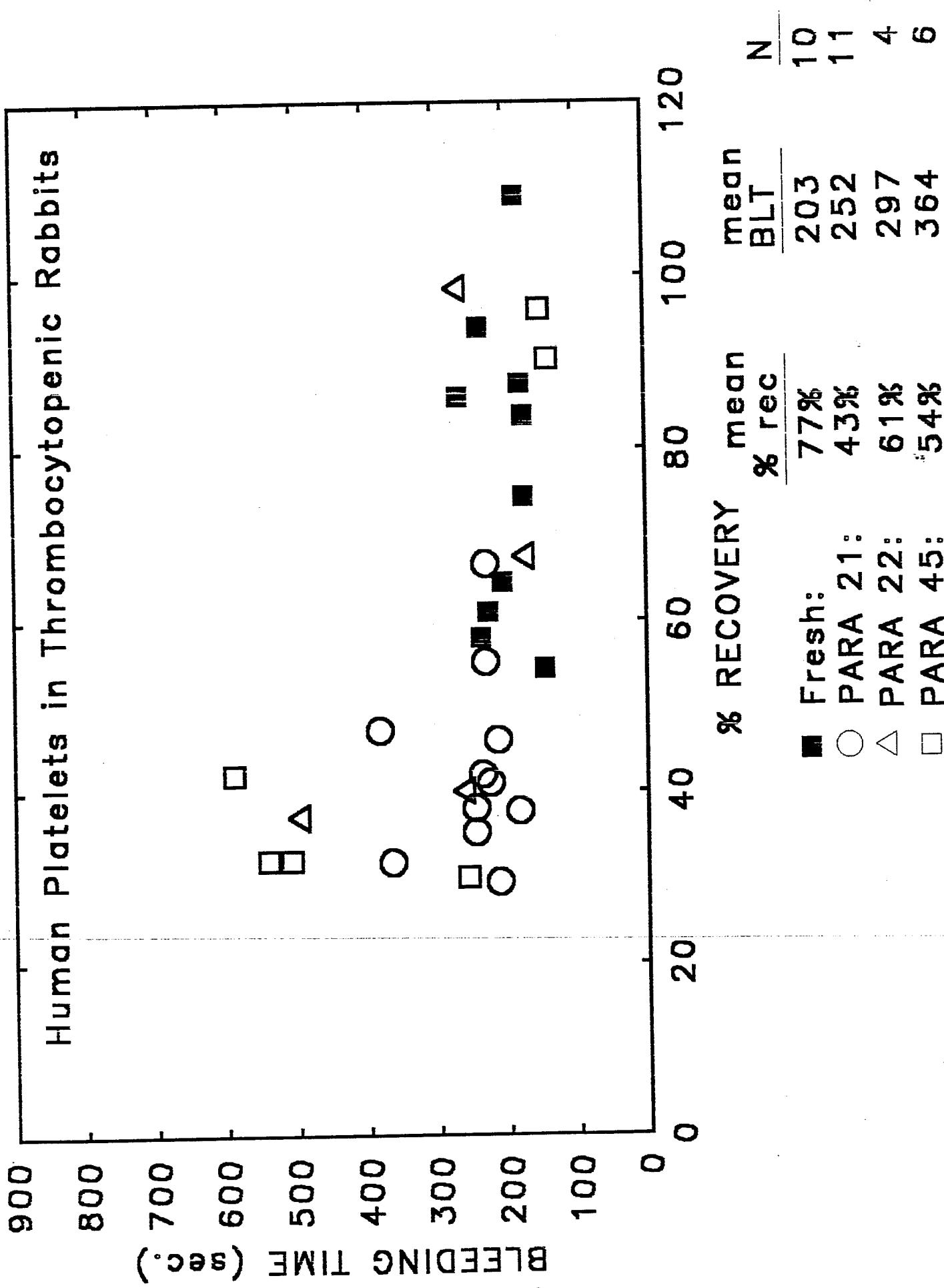
The remainder of this report is comprised of summaries of data from the ECU worksite or UNC-Chapel Hill (see subcontractor's report). At ECU, our main focus in Year Three was on preparation of platelets for testing in Blajchman's model, calcium flux measurements in rehydrated platelets as a sign of integrity of responsiveness pathways, a final assessment of alternative processing procedures such as permanganate vs. paraformaldehyde stabilization, and an initial investigation into the role of platelet-derived microparticles (PDMP) in adhesion of rehydrated platelets to thrombogenic surfaces.

Thrombocytopenic Rabbit Model of Hemostasis: The collaboration with Dr. Blajchman to test our lyophilized platelets in his model of bleeding time correction in immunocompromised Thrombocytopenic rabbits included 32 studies of freeze-dried or fresh human platelet preparations. Three different kinds of lyophilized preps were sent to Dr. Blajchman's lab in Hamilton, Ontario: fifteen separate PARA21 lots (stabilized with 1.8% paraformaldehyde for 1 hour prior to freeze-drying), four separate PARA22 lots (1.8% paraformaldehyde for 2 hours), and six separate PARA45 lots (1.8% paraformaldehyde for 45 minutes); fresh human platelets from his local sources were used as controls. For these studies, the rabbits were treated with whole body irradiation and anti-thrombocyte antiserum to reduce the platelet count to $< 20,000/\mu\text{L}$ and prolong the ear bleeding time to > 900 seconds. One hour after infusion of test platelets, the ear bleeding time and circulating platelet count increment was measured to assess the effect and survival of the infused platelets. This model has been used by Dr. Blajchman in various formats for two decades to obtain information about platelet physiology and senescence. The fact that human platelet preparations can be evaluated in this model after use of immunocompromising drugs (ethyl palmitate) has only recently been established, but has already proven useful.

The results Dr. Blajchman obtained included several interesting findings. As a whole, the lyophilized human platelets gave poorer recoveries in circulation after 1 hour than the control of fresh human platelets ($\sim 45\%$ vs. 77% mean recovery). However, when slightly larger infusions of rehydrated platelets were made to achieve the same circulating platelet count as with the controls, the PARA21 platelets gave a mean bleeding time not significantly different from fresh human platelets (252 seconds vs. 203 seconds), while the PARA22 and PARA45 gave longer bleeding times (297 and 364 seconds, respectively). These data suggest that PARA21 platelets provide good hemostatic potential which may excel that of the other preparations but is comparable to fresh platelets. The caveat of poor circulatory recoveries in this model does not seem to be particularly negative since it relates to xenographic infusions. In one set of experiments, four PARA21 preparations were left pooled in the rehydrated state inadvertently for more than four hours before infusion into subject animals; the results showed remarkably longer bleeding times (400 - 600 seconds) after these were infused compared to the fresher PARA21 infusions, leading us to suspect that the platelets had deteriorated on standing and thus should be excluded from statistical analysis (not represented in the attached charts). However, a positive spin on these



Human Platelets in Thrombocytopenic Rabbits



data would suggest such deterioration shows that the PARA21 platelets are much more like fresh than fixed, inert platelets.

Calcium Flux: We have had as a goal for many years the demonstration of an activation response in the lyophilized platelets with the aim of modifying the stabilization process to balance the trade-off of physical integrity with metabolic responsiveness. The activation of platelets involves a metabolic burst associated with mobilization of Ca^{2+} from internal stores and ion flux across the surface membrane. Paraformaldehyde fixation of the platelets prior to lyophilization may abrogate this functionality to some degree. We have determined that the minimal necessary fixation conditions are 1.8% paraformaldehyde for 60 minutes to maintain physical integrity, and are now in the process of assessing the degree of compromise of responsiveness incurred.

Our first attempts to measure calcium flux in fresh versus lyophilized platelets were with the photoprotein aequorin which gives a luminescence signal when complexed with Ca^{2+} . Loading aequorin into platelets requires a membrane permeabilization step, which we found to be difficult to put into routine practice. The few times this was accomplished without lysis of the platelet sample we found little or no signal in the lyophilized platelets on our PICA lumi-aggregometer when stimulated with collagen, ADP, or other agonists. However, with recent advances in ion-sensitive fluorescence dye technology we have reopened this investigation using flow cytometry as the detection system. The new approach involves loading platelets with Fluo-3 and Fura Red, which do not require a troublesome membrane permeabilization step. The signal sensitivity is enhanced by measuring the ratio of fluorescence between Fluo-3 and Fura Red (two different emission wave lengths, but both excited by the 488 nm laser line, Fluo-3 increases in intensity when complexed with Ca^{2+} while Fura Red decreases in intensity) and can be corrected for differential loading. With this new technique, we have found in experiments thus far that two different preparations of lyophilized platelets responded maximally in Ca^{2+} flux reproducibly (comparable to fresh platelets) with stimulation by ionophore A-23187. These results set the stage for similar experiments using physiologic agonists such as collagen, thrombin, or ADP. This work will continue during the project extension period.

Permanganate vs Paraformaldehyde: We developed in the initial phases of this project an alternative method for stabilizing platelets pre-lyophilization with 0.01 - 0.02% permanganate instead of paraformaldehyde. This year we concluded that the KMnO_4 -platelet preparations do not perform as well as PARA21 platelets in many critical tests, including hypotonic shock response, Clot Signature Analyzer Hemostasis in vitro, and Blajchman's thrombocytopenic rabbit model. In the hypotonic shock test, platelets suspensions are exposed to a one-half volume of dH_2O ; the response in normal platelets is characterized by immediate swelling and then pumping out of the taken-up water. Typical PARA21 preparations respond at one-third to one-half the rate of fresh platelets, but none of the KMnO_4 -preps we have prepared have responded at all. Ten separate KMnO_4 -preps have been run on the Clot Signature Analyzer during this reporting period. The results demonstrate hemostatic plug formation comparable to fresh platelets and PARA21, but poor collagen interaction. In the Baumgartner

platelet adhesion assay, the KMnO₄-platelets performed as well as PARA21 and PARA22 platelets in tests of their ability to adhere to thrombogenic surfaces under high shear rates. However, when infused into thrombocytopenic rabbits in Blajchman's model of in vivo bleeding time correction, KMnO₄-platelets gave worse recoveries in circulation and less correction of the bleeding time than PARA21 preparations.

In summary, we have found that the use of KMnO₄ for stabilizing platelets prior to lyophilization is successful in preserving structural integrity, but induces more of a compromise of platelet responsiveness or in vivo function than does paraformaldehyde. Thus, KMnO₄ is not readily acceptable as an alternative technology for processing transfusible lyophilized platelets. The need for an alternative processing method is not acute. Armour Pharmaceutical Corp. is proceeding with developing a large-scale GMP approach to making PARA21 platelet preparations, and paraformaldehyde seems to have more advantages than disadvantages as a component of this system. We have been developing KMnO₄ and other alternative stabilization protocols in case a toxicological risk or a serious problem in up-scaling precluded the use of paraformaldehyde.

Platelet-Derived Microparticles: In other work, we and other investigators have shown that platelets release procoagulant membranous microparticles into the supernate during direct stimulation with agonists or as a result of storage in the blood bank. In current investigations, we have found that adhesion of platelets in the Baumgartner perfusion system appears to be affected by the presence of microparticles. When the plasma used in Baumgartner experiments is passes through a 0.2 μm filter prior to perfusion, the extent of vessel surface area covered by adherent platelets is reduced 10 - 30%. Also, in the Clot Signature Analyzer the prior filtration of plasma has a marked inhibitory effect on platelet plug formation, clot generation, and collagen interaction. These findings hold for fresh or stored platelets, and are more pronounced when plasma from stored blood bank concentrates is used in the test, since this material typically has a high content of microparticles before filtration. Certain controls remain to be run, but it appears that the major effect of this filtration is the removal of microparticles from the plasma.

We have now run several rehydrated platelet preparations in filtered or non-filtered plasma in the Clot Signature Analyzer to see if removal of microparticles diminishes their responsiveness also. The results thus far show that PARA21 platelets have impaired plug formation, give a longer in vitro bleeding time, and lose response to collagen in the presence of filtered plasma. More work needs to be done to confirm these findings in the Baumgartner assay, as well as to establish the mechanism of interaction between platelets and microparticles and its significance in relation to normal hemostasis. It is reassuring that PARA21 lyophilized platelets appear to be subject to this interaction, also, since it may be an important physiologic regulator.

SUBCONTRACT REPORT FROM UNC-CHAPEL HILL

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"Evaluation of Dried Storage of Platelets for Transfusion:
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Grant No. N00014-92-J-1244
From the Office of Naval Research:
Navy Medical Research and Development Command
Department of the Navy

Performance Sites and Investigators:

University of North Carolina at Chapel Hill
Marjorie S. Read, Ph.D., Robert L. Reddick, M.D., Co-Investigators

1994 Annual Report
University of North Carolina at Chapel Hill

Contract: UNC/ECU
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Department of the Navy

Performance Site: University of North Carolina at Chapel Hill
Principal Investigator: Marjorie S. Read, Ph.D
Co-PI: Robert Reddick, MD
Submitted: March 6, 1995

Grant No. 0014-92-J-1244

March 3, 1995

Annual Progress Report: Rehydrated Platelet (RP) Studies During the Last Year.

1. Production of Animal Models of Thrombocytopenia.

During 1993, we were given an outdated and out-of-use COBE Spectra Blood Cell Separator apheresis machine. We expected to use the COBE Spectra Blood Cell Separator to render dogs thrombocytopenic in the absence of chemicals and irradiation. We successfully reduced platelets in one dog to less than 50,000 platelets/ μ l, but the bleeding time was still in the normal range of 4 min. The long time required for this procedure and the need for anesthesia made this procedure less than desirable for use with the dog and the pig. We have experiments planned for the pig using the COBE but the use of the COBE will be limited by the lack of plastic supplies. The manufacturer has discontinued this model and no replacement supplies are being produced.

To meet the need for a small animal model of thrombocytopenia that would not require large volumes of platelets, we have produced a rat model. We used a rabbit anti-rat thrombocyte antibody to produce rats with reduced levels of circulating native platelets. For study of hemostatic and thrombogenic effects of RP, a rat model of thrombocytopenia is ideal. The advantages of using a rat model are several. Due to the small size of the rat, smaller numbers of platelets are needed to attain 100% circulating RP, care and handling of the animals are minimal and inexpensive, availability is no problem, and antibodies are commercially available to induce thrombocytopenia in rats. Circulation studies in normal rats using fluorescent RP are in progress and bleeding time studies in thrombocytopenic rats have been performed.

The reduction of circulating rat platelets is dose dependent (see Table 1), making it possible to perform hemostatic studies of RP and to evaluate the interaction of RP with reduced levels of native fresh platelets. Reduced levels of circulating native platelets more closely resemble thrombocytopenia seen in the clinical setting. We use the rat model to test the hemostatic ability of our RP. Some of these data are published (see the enclosed manuscript, Read et al., PNAS, 1995.) Thrombosis studies in the normal and thrombocytopenic rat using a modification of published stenosis/injury models are being done to further characterize the rat model of thrombocytopenia. We are also using the rat model developed for these hemostatic studies for the testing of platelet preparations produced by Armour Pharmaceutical Company. That work is being performed with support from grant No. N-00014-94-C-0072, in an effort to generate a commercially available dried platelet for human platelet transfusion.

Table 1. Dose dependence of anti-rat thrombocyte antibody treatment in the rat.

platelet count pre Ab treatment	Pre BT (minutes)	Ab dose (ml)	platelet count post Ab treatment	BT post Ab treatment (minutes)
795,000/ μ l	1.5	0.5	475,000/ μ l	1.5
		0.5	110,000/ μ l	5.0
		0.3	20,000/ μ l	15.0

abbreviations: Ab=antibody, BT=bleeding time

2. Hemostatic Testing in the Rat Model.

In addition to producing a thrombocytopenic animal, we had to establish a reliable bleeding time test. Using an adaptation of the published canine toenail bleeding time test (BT), we developed a reliable rat bleeding time test. The range for normal rat bleeding time is 0.5 to 4.0 minutes, as measured in 20 rats. None of the normal rats have had a long bleeding time. The

standard rat tail BT test has proven very unreliable and variable. The tail BT varies with temperature and cut. There also appears to be variability in species of rat relative to tail bleeding times. We have not found such species variability with the toenail test.

Table 2 contains data published in PNAS and some unpublished data with additional rats. Rehydrated platelets have proved to be hemostatic in all the models used so far. We have infused RP into dogs, pigs, and rats, but the rat has proven to be the most convenient model to work with, yielding the most reliable and reproducible results.

Table 2. Hemostatic studies in the thrombocytopenic rat.

rat#	<u>pre Ab treatment</u>		<u>post Ab treatment</u>		<u>post infusion</u>	
	plt ct (/ul)	BT (minutes)	plt ct (ul)	BT (minutes)	plt ct (/ul)	BT (minutes)
1	1,025,000	1.5	75,000	>15	80,000	2.0
2	795,000	0.9	20,000	>15	75,000	1.0
3	833,000	1.3	nd	>15	101,000	3.5
4	685,000	0.5	25,000	>15	220,000	0.5
5	580,000	2.0	32,500	>15	237,000	1.5

Abbreviations: plt ct=platelet count, BT=bleeding time, Ab=antibody.

3. Circulation and recovery.

Fluorescent Labeled Platelets. The purpose of these studies was to find a method for labeling fresh and RP. During the past year, methods have been developed for labeling rehydrated and fresh platelets with fluorescein as well as with radioactive tags. These labels are essential for use in hemostatic and thrombogenic studies of rehydrated platelets in animals. The fluorescent dye PKH-26 from Zynaxis labels RP. Stability studies with PKH-26 labeled RP incubated in plasma at 37°C for 24 hours showed that the label remained with the platelets and very little was lost to the supernatant. Unfortunately, PKH-26 caused agglutination of fresh platelets under RP labeling conditions.

The method for labeling fresh platelets had to be developed by trial and error. We devised a method for labeling fresh platelets with the PKH-26, but the platelets appear activated even when no aggregation occurs. The procedure uses different diluents for the dying process than recommended by the manufacturer. The data suggests that PKH-26 is not the best method for labeling fresh platelets. We need a method for labeling both fresh and rehydrated platelets in order to make a valid comparison of recovery and circulation.

Radiolabeled Platelets.

We radiolabeled autologous canine rehydrated platelets with ¹¹¹In for infusion. The labeled platelets were infused into an adult female dog. Blood samples were taken at 5 minute intervals for the first 45 minutes, then once an hour until hour 6. A 2 ml whole blood sample was assayed for radioactivity at each time point. The level of radioactivity in the samples remained constant during the first 2 hours, then decreased to half the original amount after 6 hours. At hour 4, a whole body scan of the dog was performed to discern the distribution of radioactive label. Label was found throughout the body in a diffuse distribution, with a concentration of label in the liver. Label was also detected in the major thoracic vessels. A whole body scan performed at 24 hours revealed a concentration of radiolabel in the urinary tract. Platelet counts remained within normal range throughout the experiment. Unfortunately, later experiments showed that the ¹¹¹In label leaches out of the platelets into the plasma at 37°C. This suggested to us that the diffuse label seen in the dog was due to leached label, and not label residing in the platelets.

Fresh platelets have been radiolabeled with standard procedures using ⁵¹Chromium or ¹¹¹Indium. On the contrary, radiolabeling of rehydrated platelets has proved very difficult.

Radiolabels ordinarily used with fresh platelets are not satisfactory with rehydrated platelets since they are readily lost from the platelet in the presence of plasma at 37°C. We have investigated ¹¹¹Indium, ⁵¹Sodium Chromate, ¹²⁵Iodine, and ¹³¹Iodine. We have successfully labeled rehydrated platelets in saline at room temperature with ¹¹¹In. These platelets contained 90% of the label after 24 hours incubation at room temperature. However, subsequent incubation of the platelets in plasma resulted in a partial release or leaching of the radiolabel to the supernatant. Rehydrated platelets were labeled with ¹²⁵I and ¹³¹I with limited success. Label efficiency was approximately 50% with ¹²⁵I but the label was removed from the RP with washing. ¹³¹I did not label RP with a high efficiency. ⁵¹Sodium Chromate, which has been used successfully with fresh platelets, did not label RP. We have also investigated labeling RP with ⁵¹Sodium Chromate and ¹¹¹Indium prior to the fixation process, in hopes of 1) increasing the efficiency of label, and 2) stabilizing the label on the platelets. Labeling the platelets prior to fixation did not substantially increase the efficiency or stabilization of the label. These studies were presented at the American Society of Hematology meeting in Nashville in December, 1994 (see enclosed abstract.)

We have continued our work on the development of a radioactive labeling method for rehydrated and fresh platelets for use in hemostatic and thrombogenic studies. We have been successful in radiolabeling rehydrated platelets with the Zynaxis ¹²⁵IPKH-95 compound, with over 90% of the label remaining in association with platelets after 24 hours of incubation in normal dog plasma. However, the commercial production of this compound has recently been discontinued. We have obtained permission from the manufacturer to produce the compound in our own laboratory, and are currently doing so with the aid of Dr. Richard Kowalsky. Initial labeling experiments suggest that the ¹²⁵IPKH-95 remains in the rehydrated platelets when incubated in plasma at 37°C (see attached abstract, Kowalsky et al., 1994.) We are now optimizing the labeling process to maximize ¹²⁵I signal while minimizing damage to the platelet. We will be using this labeled molecule in future experiments to determine recovery and circulation.

4. Platelet Antibodies: Production of Rehydrated Platelet Specific Antibodies.

In an attempt to find other markers for fresh and rehydrated platelets, we have generated polyclonal antibodies to fresh and rehydrated human and fresh and rehydrated canine platelets. New Zealand white rabbits were immunized with platelets emulsified with Freund's complete adjuvant. Subsequent injections were performed with Freund's incomplete adjuvant every other day for 1 week. Rabbit serum was tested using ELISA and immunoblotting of platelet proteins, and by aggregation assays of fresh and rehydrated canine and human platelets. The ELISA results indicate the presence of antibodies reactive to fresh and rehydrated platelets in the rabbit antisera. Immunoblots confirm reactivity of the antibodies with fresh and rehydrated platelet lysates. However, the specificity of the antibodies in the sera is not completely characterized in that each antiserum tested reacts to some extent with both fresh and rehydrated platelet lysates. Platelet aggregation studies indicate that serum from rabbits injected with fresh human platelets aggregate fresh human platelets with a higher efficiency and at a faster rate than rehydrated platelets. Conversely, antibodies generated to rehydrated human platelets aggregate RP with a higher efficiency and at a higher rate than fresh platelets. Similar results were seen in rabbits using canine fresh and rehydrated platelets. A second set of animals has been immunized and boosted for the generation of fresh human specific or dried human specific antibodies. We are currently characterizing these rabbit sera for antibody activity.

5. Characterization of RP Surface Proteins.

The goal of these studies is to investigate and understand the interaction of surface proteins or integrins and how their interactions relate to and support adhesion and agglutination. We are also looking at changes that may have occurred as a result of the drying process and how those changes effect RP function.

SDS-PAGE/Western blotting and light microscopy is being used to localize and explore common surface antigens on rehydrated lyophilized platelets. RP and fresh platelets secrete platelet proteins when incubated in buffer at 37°C. RP and fresh platelets are disrupted by freeze-thaw and solubilization in detergent. SDS-PAGE of RP lysates and secreted proteins show a coomassie staining profile comparable to that seen in fresh platelet lysates and secreted material. Western blots using antibodies against several platelet proteins (actin, fibrinogen, fibronectin, von Willebrand factor, thrombospondin, glycoprotein (GP) Ib, and GPIIb/IIIa) suggest that lysates prepared from RP react with antibodies to actin, vWF, fibrinogen, and GPIb. Antibodies against GPIIb/IIIa, thrombospondin, and fibronectin did not react with lysates or secreted proteins from rehydrated lyophilized platelets in the western blot. This result was surprising since we have evidence by flow cytometry that these proteins are present on the RP surface. We are continuing these studies using other antibodies against the same proteins to insure that the lack of antibody interaction with specific proteins is not epitope or concentration dependent.

Light microscopy studies using antibodies to vWF, fibrinogen, actin, myosin, tropomyosin, thrombospondin, fibronectin, GPIb, and GPIIb/IIIa are also being performed in our laboratory. The antibodies are fluorescently labeled to visualize localization of proteins within a platelet adherent to a glass slide. Platelets are allowed to spread on the glass slide for 0.25-4 hours before fixation. Studies to date suggest that, although the rehydrated platelets do not spread as quickly as fresh platelets, cytoskeletal and surface proteins on rehydrated and fresh platelets appear in similar patterns. For example, actin filaments appear throughout fresh and rehydrated spread platelets with extensions into the pseudopodia. GPIb and GPIIb/IIIa proteins appear on the exterior membrane of both fresh and rehydrated platelets which have undergone minimal spreading. Our investigations on the cytoskeletal proteins of rehydrated platelets are intended to both characterize the platelet antigens on the rehydrated platelet, and to shed light on the ability or inability of a hemostatic plug formed with rehydrated platelets to contract.

6. Thrombogenicity of Rehydrated Platelets.

We have previously reported that lyophilized platelets generate thrombin at a faster rate than unactivated fresh platelets in the Prothrombinase Complex reaction. In spite of this apparent increased rate of thrombin generation, tests of plasma and serum samples from a dog transfused seven times with rehydrated canine platelets showed no significant change in factor VIII or factor IX levels. The prothrombin times for pre- and post-infusion samples were not significantly different, and no change in the production of fibrinogen degradation products was noted. The partial thromboplastin time test (PTT), a measure of the intrinsic coagulation mechanism was similar in pre- and post-infusion samples.

Publications

1. Read, MS, Reddick, RL, Bode, AP, Bellinger, DA, Nichols, TC, Taylor, KK, Smith, SV, McMahon, DK, Griggs, TR, Brinkhous, KM. (1994) Preservation of hemostatic and structural properties of rehydrated lyophilized platelets: Potential for long-term storage of dried platelets for transfusion. *Proc. Natl. Acad. Sci. USA* 92, 397-401.
2. Kowalsky, RJ, Taylor, KK, McMahon, DK, Brecher, ME, Bellinger, DA, Reddick, RL, Read, MS. (1994) A stable radiolabel for fresh and dried platelets. *Blood* 84, A1278.

Proc. Natl. Acad. Sci. USA
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Medical Sciences

Preservation of hemostatic and structural properties of rehydrated lyophilized platelets: Potential for long-term storage of dried platelets for transfusion

(platelet adhesion/platelet agglutination/thrombocytopenia/thrombosis)

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Contributed by Kenneth M. Brinkhous, September 22, 1994

ABSTRACT Currently, therapeutic platelet concentrates can be stored for only 5 days. We have developed a procedure that permits long-term storage of fixed and lyophilized platelets that retain hemostatic properties after rehydration. These rehydrated lyophilized platelets (RL platelets) restore hemostasis in thrombocytopenic rats and become incorporated in the hemostatic plug of bleeding time wounds of normal dogs as well as von Willebrand disease dogs with partially replenished plasma von Willebrand factor. Ultrastructurally, these platelets are well preserved and are comparable to control normal washed platelets. Flow cytometry analysis shows that RL platelets react with antibodies to the major surface receptors, glycoprotein (GP)Ib and GPIIb/IIIa. These receptors are involved in platelet agglutination, aggregation, and adhesion. *In vitro* functional tests document the ability of RL platelets to adhere to denuded subendothelium and to spread on a foreign surface. Circulating RL platelets participated in carotid arterial thrombus formation induced in normal canine subjects. The participation of RL platelets in these vital hemostatic properties suggests that with further development they could become a stable platelet product for transfusion.

To promote effective hemostasis, platelets must respond quickly to changes in normal blood flow or vessel injury (1, 2). After vascular injury, platelets adhere to exposed subendothelium, aggregate, and form a primary platelet plug. Platelet activation and initiation of coagulation follow with stabilization of the platelet plug by the formation of fibrin. The initiation of a thrombus at a site of vascular injury is mediated through platelet membrane glycoprotein (GP) receptors (3, 4). Platelet adhesion to a damaged vessel wall and its extracellular matrix at high shear is primarily mediated through the specific interaction of the platelet membrane GPIb-IX complex and bound von Willebrand factor (vWF) (5-7), which is synthesized and released into plasma and the vessel wall by endothelial cells (1). Platelet adhesion at low shear rates is mediated by several interactions, including collagen with the $\alpha_2\beta_1$ integrin (7). Platelet adhesion stimulates a spreading of the platelet (8). Although the mechanism of platelet spreading has not been completely characterized, recent *in vitro* studies have shown that platelets will spread on surfaces coated with fibrinogen (9) or polymerized fibrin (10). The activation of the GPIIb/IIIa receptor by agents such as ADP results in a conformational change in the receptor (11-13). The activated receptor binds fibrinogen, which forms a "bridge" between the platelets, and causes aggregation (1, 14, 15). Activated platelets provide the phospholipid surface for the assembly of blood clotting en-

zyme complexes, and the concentration and localization of activated coagulant proteins at sites of vessel wall injury may be facilitated by adherent platelets (16). Internal storage granules in platelets release clot-promoting contents in response to activation of biochemical systems triggered by platelet-platelet or other interactions. Interactions of adherent platelets with neutrophils, mediated through platelet integrins, specifically P-selectin receptor (17), may contribute to hemostatic and other cell functions (13).

The control of hemorrhage due to thrombocytopenia often requires transfusion of multiple units of fresh platelets. In transfusion medicine, platelets cannot be replaced by other blood products or artificial media. Maintenance of critical membrane GPs during storage is crucial to platelet function *in vivo*. With the storage life of fresh platelets limited to 5 days, there has been considerable study to lengthen platelet shelf life and enhance stored platelet response (18-24). Investigators have addressed storage conditions that preserve platelet integrity and responsiveness (25, 26). The effects of preservatives on platelet activation and expression of membrane GPs have also been investigated (27). In a recent review, the use of inhibitors of platelet activation to extend the shelf life and enhance the quality of liquid stored platelets is discussed (28). Cryopreservation of platelets extends the shelf life to 1 year but requires extensive washing and processing to remove cryoprotectant agents (29). In other blood cell studies, red blood cells washed with saline followed by lyophilization retain metabolic activities similar to red blood cells stored under blood bank conditions (30). Lyophilization of platelets or platelet-rich plasma as previously attempted (31-33) neither preserved the structural integrity of the platelets nor provided adequate hemostasis when infused into thrombocytopenic pediatric patients or hemorrhagic animal models.

We have successfully prepared a paraformaldehyde-treated, lyophilized and rehydrated platelet product (RL platelets) with intact morphology and agglutinating properties (34-36). Efforts to refine this process have produced platelet preparations that are structurally stable and capable of undergoing activation. We report here on the hemostatic properties displayed by our RL platelets as tested *in vitro* and in canine and rat animal models. In this study, we have administered RL platelets to normal and von Willebrand disease (vWD) dogs and to thrombocytopenic rats. Our results indicate that RL platelets retain many essential biologic properties and promote hemostasis. The successful preparation of a dried transfusion platelet product without loss of hemostatic capabilities

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Abbreviations: RL platelet, rehydrated lyophilized platelet; BT, bleeding time; vWD, von Willebrand disease; vWF, von Willebrand factor; GP, glycoprotein.

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suggests potential for the development of this product as a blood banking resource.

MATERIALS AND METHODS

Preparation of RL Platelets. Lyophilized human and canine platelets were prepared as described (34). Human platelets were obtained from the American Red Cross 3–6 days after collection. Canine platelets were obtained from normal dogs ($n = 5$) from the Francis Owen Blood Research Laboratory (University of North Carolina). Washed platelets were incubated for 1 hr with paraformaldehyde at concentrations of 1.8% for human platelets and 0.68% for canine platelets. Washed paraformaldehyde-free platelets in citrated saline (0.006 M trisodium citrate/0.154 M NaCl, pH 6.8) with 5% bovine serum albumin were frozen in 1-ml aliquots containing 8×10^8 platelets per ml and lyophilized at -20°C to -40°C for 20–24 hr. Dried platelets were stored at -80°C until used. Dried platelets were rehydrated in 1.0 ml of imidazole buffer (IB; 0.084 M imidazole, pH 7.35) and centrifuged at $1000 \times g$ for 8 min to pellet the platelets. The rehydrated platelets were freed of albumin and imidazole by three washes in citrated saline. For use, the platelet pellets were resuspended in platelet-poor plasma or in a modified Hanks' buffered salt solution (mHBSS; 0.17 M NaCl/6.7 mM KCl/1.0 mM MgSO₄/0.5 mM K₂HPO₄/2.8 mM Na₂HPO₄/13.8 mM dextrose, pH to 7.2 with 1.4% NaHCO₃) for *in vitro* studies and in normal saline for *in vivo* studies. Gas chromatography was used to document the absence of formaldehyde in washed canine and human RL platelet solutions. A detection limit of 0.002% was used (National Medical Laboratories, Willow Grove, PA).

RL platelets were labeled with the fluorescent dye Zynaxis PKH 26 as a marker for platelets in infusion studies. The pelleted rehydrated platelets were washed once by resuspension in 1.0 ml of acid citrate dextrose, centrifuged at $600 \times g$ for 8 min, and resuspended in 0.1 ml of mHBSS. The mixture was incubated for 20 min in the dark and centrifuged ($600 \times g$; 8 min). The labeled platelets were washed once by resuspending in 1.0 ml of mHBSS containing either 0.1% canine serum albumin or 0.1% bovine serum albumin. No label was transferred or lost from fluorescent platelets incubated at 25°C – 37°C for several hours in whole blood.

Hemostatic and Other Methods. The saline bleeding time (BT) in canines was performed as described (37). The BT wound sites were excised and prepared for fluorescence and light microscopy (38). For the rat toenail BT, rats were anesthetized with ketamine hydrochloride (Ketasert)/ProMACE, and a foot was antisepically cleansed and warmed in a 37°C bath. A sterile scalpel blade was used to excise the distal 1.0 mm of the vascular nail bed from one nail. Blood was blotted onto filter paper for BT measurements. Platelet adhesion of fresh and RL human platelets was compared in an annular perfusion chamber (39). Adhesion studies were carried out at high shear (flow rate, 125 ml/min; 37°C) using porcine arterial subendothelium that had been denuded by exposure to air. After platelet solutions containing fresh or RL platelets were exposed to the subendothelium, segments were removed and processed for scanning electron microscopy to visualize platelet adhesion. Citrated blood, platelet-rich plasma, platelet-free plasma (PFP), and red blood cell fractions were isolated as described (34). Adhesion of fresh platelets was determined after passing whole citrated blood over the subendothelium. Adhesion of RL platelets was determined after PFP enriched with RL platelets and the red blood cell fraction was passed over the subendothelium. To confirm the absence of platelets in PFP prior to the addition of RL platelets, phase-contrast microscopy was used. To examine platelet spread, RL platelets were reconstituted in IB, washed once in HBSS (40) to remove albumin, and spread on Formvar-coated grids (41). Spread platelets were examined

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with a Cambridge autoscan scanning electron microscope at 20 kV.

For morphological studies, fresh and RL platelet pellets were processed for transmission electron microscopy as described (38). Platelets were examined with a Zeiss 10A microscope. Rehydrated platelet surface antigen distribution and overall light scatter properties were analyzed on a Becton Dickinson FACS 440 flow cytometer. Monoclonal antibody binding to rehydrated platelets or to fresh platelets resuspended in citrated plasma was evaluated by indirect immunofluorescence 488-nm excitation as described (42). Control antibody was used to identify nonspecific IgG binding to fresh and rehydrated platelets. In each run, 10,000 events were measured and analyzed.

Infusions of RL Platelets. The animals used were normal dogs ($n = 3$), a vWD dog ($n = 1$), and Sprague-Dawley rats ($n = 3$). The vWD dog was from the closed colony at the Francis Owen Blood Research Laboratory. Normal Sprague-Dawley rats were obtained from the Division of Laboratory Animal Medicine (University of North Carolina, Chapel Hill). All animals were treated according to published standards (43). Thrombocytopenic rats were given 4.5×10^9 and 3.4×10^{10} human RL platelets, respectively, through the tail vein. Toenail BTs were performed immediately after infusion of RL platelets. Normalization of BT was taken as an indicator of RL platelet hemostatic function.

Fluorescence-labeled canine RL platelets were infused into three normal dogs. After infusion of these platelets, plasma levels of vWF (44), coagulation factor IX (45), coagulation factor VIII (46), platelet counts (Unopette; Becton Dickinson), and serum fibrin degradation products (ThromboWellcotest; Wellcome) were determined prior to and at the

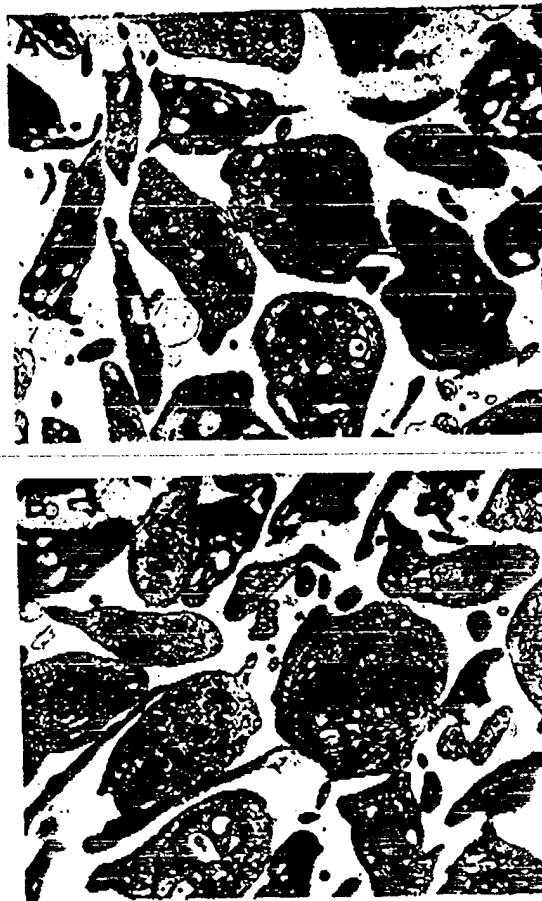


FIG. 1. Transmission electron microscopy of fresh (A) and RL (B) platelets. Both fresh and RL platelets have intact and randomly distributed organelles and some pseudopod formation. ($\times 7760$)

Table 1. GPIb, GPIb/IIIa, and GPIb/IX on the surface of fresh and RL platelets

Antigen	Antibody	% labeled platelets	
		Fresh	RL
GPIb	AN-51	98	92
GPIb	SZ-2	92	85
GPIb/IX	SZ-1	98	92
GPIIb/IIIa	10E5	98	98

Monoclonal antibodies to GPIb (clones AN-51 and SZ-2) were obtained from Dakopatts (Glostrup, Denmark) and AMAC (Westbrook, ME), respectively. The anti-GPIb/IX complex antibody (SZ-1) and the anti-GPIIb/IIIa complex antibody (clone 10E5) were obtained from AMAC and Barry Collier (State University of New York, Stony Brook), respectively. There were <5% labeled platelets in the positive gates using a control nonimmune mouse IgG-2a antibody (Coulter Immunology). Fresh platelets were washed with citrated saline.

following intervals postinfusion: 1, 5, 15, 30, and 60 min during the first hour, and 2, 4, 6, 8, and 24 hr thereafter. BTs were performed postinfusion of labeled RL platelets and wounds were excised after cessation of bleeding for examination by fluorescence and light microscopy (38). A similar infusion was

performed with a vWD dog after partial replacement of vWF by treatment with cryoprecipitate. RL platelets represented 51.4% of the vWD dog's normal platelet count based on the number of RL platelets infused. Cryoprecipitate was prepared as described (47) and assayed for vWF content (44). A modified Fols procedure (48) was used to produce carotid arterial thrombosis in dogs ($n = 3$).

RESULTS AND DISCUSSION

Structural Features of RL Platelets. Transmission electron microscopy showed that RL platelets are morphologically similar to fresh washed platelets (Fig. 1). RL platelets are partially activated, similar to fresh washed platelets. Flow cytometry using anti-GPIb and anti-GPIIb/IIIa monoclonal antibodies indicated that both GPs were present on the surface of RL platelets (Table 1). The number of RL platelets with antibody recognition of receptors is expressed as a percentage of the platelets with specific immunofluorescence.

Functional Characteristics of RL Platelets *in Vitro*. Platelet adhesion and platelet spreading are shown in Fig. 2. A comparison of platelet adhesion with fresh and RL platelets shows that RL platelets adhere in numbers similar to fresh platelets, with irregular shapes and with multiple pseudopodia.

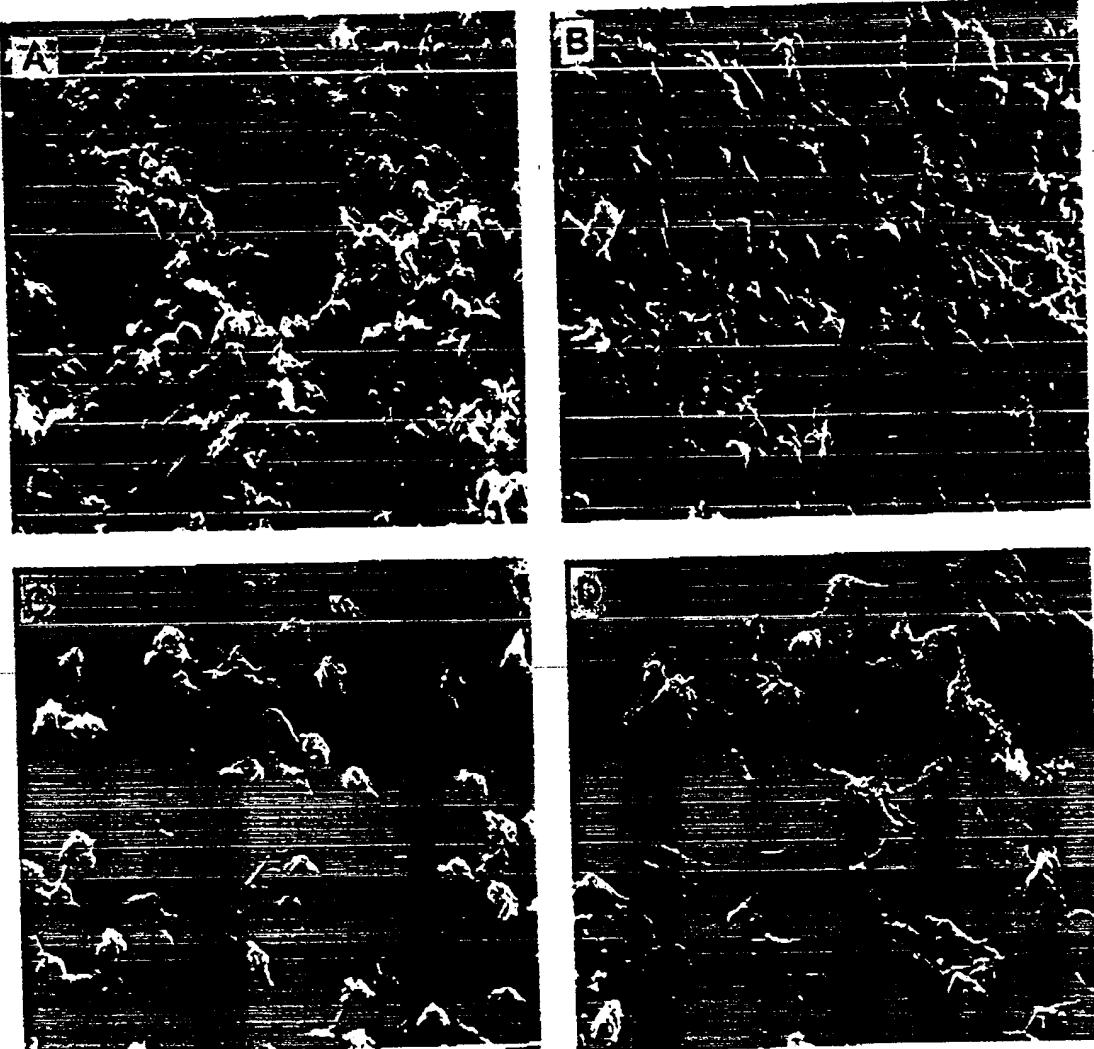


FIG. 2. Scanning electron microscopy (SEM) of vessel segments from an annular perfusion chamber with RL platelets (A) and fresh platelets (B). Vessel subendothelium exposed to platelet-free blood was free of platelets (data not shown), while segments exposed to platelet-containing blood was carpeted with platelets. SEM of spread RL platelets shows that paraformaldehyde-stabilized platelets adhere to Formvar-coated grids formed dendritic patterns with multiple pseudopodia and are fully spread (C), similar to fresh platelets spread on the same substrate (D). (A, $\times 2320$; B, $\times 2320$; C, $\times 2080$; D, $\times 1600$.)

Table 2. Infusions of human RL platelets shorten the prolonged BTs in thrombocytopenic rats

Animal	Normal		Thrombocytopenic		Thrombocytopenic with RL platelets	
	Platelet count	BT. min	Platelet count	BT. min	Platelet count	BT. min
	per μ l. $\times 10^{-3}$		per μ l. $\times 10^{-3}$		per μ l. $\times 10^{-3}$	
1	0.5	685	>15	25	0.5	220
2	2.0	580	>15	32.5	1.5	237

Thrombocytopenia was induced in two Sprague-Dawley rats by treatment with 1 ml of a 1:10 dilution of anti-rat thrombocyte polyclonal antibody (Accurate Chemicals). Platelet counts and toenail BT measurements were used to monitor the level of circulating rat platelets 10 min after treatment with the antibody. RL platelets were infused immediately after a BT of >15 min was established. In a control rat without treatment with RL platelets, bleeding times were >15 min, and platelet counts were <50,000 platelets per μ l for >12 hr.

although pseudopodia are present to a lesser extent in RL platelets (Fig. 2A and B). Neither RL platelets nor fresh platelets were present in areas where the endothelium remained intact (data not shown). A comparison of platelet spreading of fresh and RL platelets showed both having a similar flattened or "pancake" morphology (Fig. 2C and D). Multiple pseudopodia were found associated with both fresh and RL platelets, which were not completely spread. Few

discoid forms were present, suggesting that paraformaldehyde-stabilized platelets retained sufficient metabolic activity for platelet spreading to occur. Earlier studies have demonstrated that functional GPIb is preserved in lyophilized platelets (49). While GPIIb/IIa epitopes are identified in RL platelets, minimal platelet aggregation was observed in preliminary studies with ADP (A.P.B., unpublished data).

Functional Characteristics of RL Platelets in Vivo. RL platelets were labeled with a fluorescent dye to distinguish rehydrated platelets from circulating native platelets in infusion experiments. BT studies in rats with human RL platelets and in normal and vWD dogs with canine RL platelets were conducted. The results of two separate RL platelet infusion experiments using thrombocytopenic rats are shown in Table 2. After administration of human RL platelets, toenail BTs in two rats treated with an anti-rat thrombocyte antibody decreased from >15 min to normal. One rat was tested at 30 min and had a toenail BT of 3.5 min, which remained corrected for 1 hr, at which time the rat was sacrificed. Treatment of normal rats with diluted anti-rat thrombocyte antibody depleted circulating rat platelets to <33,000 platelets per μ l and lengthened the rat toenail BT to >15 min. Normal rats treated with experimental levels of the anti-rat thrombocyte antibody without additional RL platelets had elongated BTs (>15 min) and low autologous platelet counts (<50,000 platelets per μ l) for 12 hr. The toenail BT is a simple and reproducible method of measuring BT in the rat and requires only that the rat be anesthetized before testing. In 10–12 normal rats, the BTs ranged from 30 sec to 3 min.

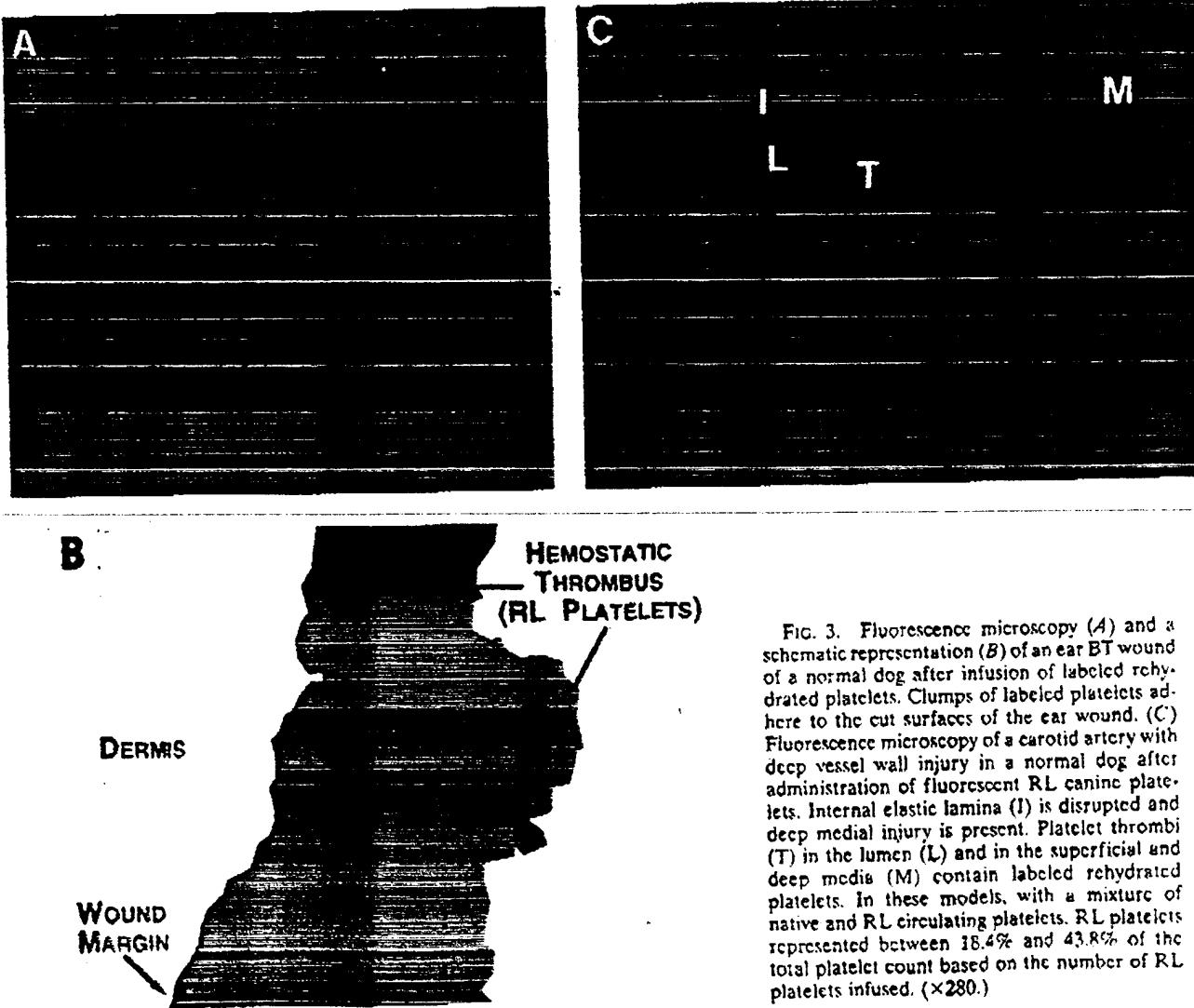


FIG. 3. Fluorescence microscopy (A) and a schematic representation (B) of an ear BT wound of a normal dog after infusion of labeled rehydrated platelets. Clumps of labeled platelets adhere to the cut surfaces of the ear wound. (C) Fluorescence microscopy of a carotid artery with deep vessel wall injury in a normal dog after administration of fluorescent RL canine platelets. Internal elastic lamina (I) is disrupted and deep medial injury is present. Platelet thrombi (T) in the lumen (L) and in the superficial and deep media (M) contain labeled rehydrated platelets. In these models, with a mixture of native and RL circulating platelets, RL platelets represented between 18.4% and 43.8% of the total platelet count based on the number of RL platelets infused. ($\times 280$.)

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RL platelets were infused into three normal dogs and one vWD dog in order to determine whether the RL platelets were incorporated into the hemostatic thrombi of BT wounds. The vWD dog was infused with cryoprecipitate, which raised the vWF level to 38% of normal; the BT of the vWD dog was reduced from >15 min to 8 min. RL platelets were then administered to the vWD dog, and there was no significant change in the BT. The BT remained in the normal range (6 min) for the other three dogs. RL platelets circulated for the duration of the experiments (up to 4 hr). They were found to be part of the hemostatic plug in normal dogs (Fig. 3A and B). In the vWD dog, RL platelets were observed at the same sites as in the normal dogs but in far fewer numbers (data not shown). Samples of liver, lung, and kidney were examined after sacrifice of these animals. No gross or microscopic changes were observed.

Carotid arterial thrombosis was induced by using a canine model of stenosis and injury in three normal dogs that had been infused with RL platelets (48, 50). All animals experienced occlusive thrombosis as indicated by the cessation of blood flow. When thrombosis occurred, the vessels containing the thrombi were harvested after >30 min of observation and examined by fluorescence microscopy. Fluorescent RL platelets were present in the induced thrombi and were also adherent to the exposed subendothelium. Single and aggregated platelets were present in areas of hemorrhage (Fig. 3C). In vessels where damage was minimal, fluorescent RL platelets were seen in the lumen and adhering to the luminal surface where the internal elastic lamina was disrupted. There was no evidence of RL platelets adhering to intact endothelium. The absence of disseminated intravascular coagulation was indicated by no change in fibrinogen level, no loss of coagulation factors VIII and IX, and no appearance of fibrin degradation products.

Platelet preparations can be stored for several days without the use of refrigeration. After this period, platelets lose many of their functions (51, 52). Lyophilization has the potential to extend blood cell (erythrocytes and platelets) shelf life from days to years. The fixation of platelets in paraformaldehyde followed by lyophilization has proven effective in maintaining some of the normal functions of the human platelet (34). We have shown that RL platelets retain many properties necessary for normal hemostasis. These observations suggest that this method of stabilization may offer a method for long-term storage of platelets.

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PROMOTION OF PLATELET ADHESION TO THROMBOGENIC SITES BY MICROPARTICLES IN STORED PLATELET CONCENTRATES. L.Y. Yang* and A.P. Bode, Department of Pathology, East Carolina University, Greenville, NC.

During storage of platelet concentrates (PC) in the blood bank, platelet-derived microparticles (PDMP) appear in the supernatant plasma. The hemostatic value of PDMP in transfusions has yet to be determined, in part because of the difficulty in defining and purifying PDMP. We have carried out a qualitative test of PDMP function in the Baumgartner perfusion chamber (Baum) and in a prototype bleeding time device called the Clot Signature Analyzer (CSA) (Xylum Corp, NY) by comparing results with PDMP-rich supernatant plasma from outdated PC before and after removal of PDMP by filtration of the plasma through a 0.2 micron Acrodisc filter. This filtration lowered PDMP counts as assessed by flow cytometry by > 90%. The Baum experiments were performed with fresh platelets or platelets from expired PC resuspended in filtered or non-filtered plasma from expired PC, mixed with an equal volume of washed RBC. The CSA experiments were performed on the same recombined whole blood samples immediately after recalcification with 5 mM CaCl₂. The results from the Baum runs were quantified as the percent of coverage of surface area of the denuded vessel strip in the chamber as revealed by immunofluorescence of adherent platelets under epifluorescent microscopy. The CSA measures platelet adhesion and hemostatic plug formation in vitro with several parameters, designated H1, H2, and IVBT (in vitro bleeding time); platelet procoagulant activity and collagen interaction are related to the parameters designated CT1, CT2, and CITF (collagen-induced thrombus formation). Mean results (n=9) for selected parameters are presented below:

	BAUM % Coverage	CSA IVBT	CSA CITF	PDMP x10 ⁶ /mL
Non-Filtered	60%	99 sec.	68%	62
Filtered	34%	215 sec.	51%	5
paired t-test	p=0.01	p=0.07	p=0.04	p=0.004

For all parameters, platelets in PDMP-poor filtered plasma gave values signifying poorer adhesion or function versus the same platelets in PDMP-rich non-filtered plasma. H2 and CT1 did not achieve statistical significance in the paired t-test because of a high degree of variance in the determinations. However, in the Sign test all differences showed statistical significance at p ≤ 0.05. We believe these findings demonstrate a positive effect of PDMP on adhesion of platelets and their hemostatic function as assessed in these tests.

Lyophilized platelets included on poster board of CSA results.

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A STABLE RADIOLABEL FOR FRESH AND DRIED PLATELETS. RJ Kowalsky, KK Taylor, DK McMahon, ME Brecher, DA Bellinger, RL Reddick, MS Read. University of North Carolina, Departments of Pathology, Pharmacy, and Radiology, Chapel Hill, NC.

In previous studies dried rehydrated platelets retained hemostatic properties and behaved like fresh platelets in animal models (PNAS submitted). However, dried platelets, unlike fresh platelets, did not retain a radiolabel. A stable radiolabel has been developed for fresh and stabilized lyophilized rehydrated canine platelets. ^{111}In -Tropolone, ^{51}Cr -Sodium Chromate, ^{125}I -Iodogen with Iodogen or Iodobeads, and ^{125}I -PKH95 were examined regarding labeling efficiency (LE), label stability over time in normal dog plasma or buffer, and botrocetin stimulation following radiolabeling. The table below indicates that ^{111}In -Tropolone and ^{125}I -PKH95 produced the highest LE. The high LE of ^{111}In -Tropolone, however, is compromised by poor label stability during incubation in NDP, with label translocation from platelets to plasma protein. The low LE and instability of ^{51}Cr and ^{125}I -Iodogen labels make them unsuitable platelet labels. Only ^{125}I -PKH95 provides satisfactory LE and label stability. Higher LE is achieved when Mfr supplied buffer is used during labeling, however, this buffer causes aggregation of fresh platelets. Mfr buffer did not affect the LE, stability or botrocetin-induced aggregation of dried platelets. Substitution of PBS as the labeling buffer with ^{125}I -PKH95 lowered the LE but did not promote aggregation of fresh platelets during the labeling reaction. Both fresh and dried canine platelets labeled with ^{125}I -PKH95 in PBS demonstrate stable radiolabeled platelets that undergo botrocetin stimulated aggregation following labeling and warrants further consideration as a label for in vivo platelet survival studies.

Radiolabel	Percent LE		Percent Bound Over Time *	
	Fresh	Dried	Fresh	Dried
^{111}In -Trop	94	88	98 (16 hr)	B
^{111}In -Trop	97	88	73 (20 hr)	N
^{51}Cr -Sod Chr	19-22	0	28 (21 hr)	N
^{125}I -Iodogen	2-9	19	46 (18 hr)	N
^{125}I -Iodobeads	7-23	3	30 (18 hr)	N
^{125}I -PKH95 (Mfr Buffer)	--	89-94	--	91 (22 hr)
^{125}I -PKH95 (PBS)	67	65	93 (23 hr)	N
				89 (72 hr)
				97 (24 hr)

* N = normal dog plasma @ 37°C B = ACD/Saline Buffer @ Rm Temp

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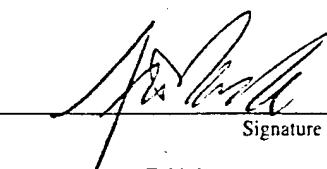
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